

REMARKS

Claims 9, 20-21, and 23-24 have been amended for clarification purposes and to correct informalities. No change in scope is intended by way of these amendments. New claim 25 depends from claim 9 and specifies a leaf-specific promoter. Support for new claim 25 is found in the specification and in claims 12 and 17, as originally filed.

Claims 9 and 20-24 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. Initially, the Examiner finds that the term "gene" of claims 9 and 23 is unclear. According to the Examiner, the term "gene" implies a DNA sequence that exists in nature and includes coding and noncoding regions, as well as regulatory sequences associated with expression. The Examiner recognizes that this is not applicants' intention and suggests alternative language. Applicants respectfully submit that the term "gene" may be used to in other ways, for example the portion of the DNA that is transcribed into RNA. (See, e.g., MOLECULAR BIOLOGY OF THE CELL (3<sup>rd</sup> ed., pg 423)). However, in an effort to advance prosecution and to clarify the claim language, the term "structural gene" has been replaced with "coding sequence of a structural gene" and the term "gene encoding a site-specific recombinase" has been replaced with "coding region encoding a site specific recombinase." These terms have been amended accordingly throughout the claims.

The Examiner has further rejected claim 9 because the preamble recites "compounds" when only one compound is produced. Claim 9 has been amended to replace "commercially valuable compounds" with "a compound."

The Examiner also rejected claim 9 because the term "the transgenic plant" in line 8 lacks antecedent basis. Applicants respectfully disagree. In line 3, claim 9 recites "a fertile transgenic plant." However, to clarify that these are the same transgenic plants, line 9 has been amended to recite "the fertile transgenic plant."

The Examiner additionally rejects claim 9 because it is unclear if the term "fertilizing" refers to adding a nutritional supplement or to pollination. The term "fertilizing" has been replaced with "pollinating."

The Examiner finds that the term "the recombinase" of claim 9, line 14 lacks antecedent basis. Applicants respectfully disagree. Claim 9, line 12 recites "a site-specific recombinase." However, to clarify that these are the same recombinases, applicants have amended "the recombinase" to read "the site-specific recombinase."

Finally, the Examiner finds that the term "economical quantities" is unclear because the metes and bounds of the claim cannot be determined. Applicants respectfully

submit that the term "economical quantities" refers to a compound that is produced in sufficient quantities that the value of the extracted compound exceeds the costs associated with standard production and extraction methods. Because the compound is detrimental to the plant, expression must be controlled by the methods of the present claims.

Claims 9 and 20-24 have been amended to clarify the claim language.

Accordingly, applicants respectfully request withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

Claims 9 and 20-24 stand rejected under 35 U.S.C. § 112, first paragraph.

According to the Examiner, the specification, while being enabling for a method of producing a compound that is not detrimental to the plant, is not enabling for a compound that is detrimental to the plant. According to the Examiner, the state of the art is that one skilled in the art can readily make DNA constructs containing a structural gene encoding a compound, transform these into plants, and express them with a reasonable expectation of success. However, according to the Examiner, expressing a compound detrimental to the plant, such as barnase, is more unpredictable, and that expression of a gene encoding such a detrimental compound must be regulated in order to avoid killing all cells expressing the compound. Further, according to the Examiner, recombinase mediated excision of appropriately flanked DNA sequences is variable and yields chimeric phenotypes having both recombined and unrecombined DNA. The Examiner cites Gidoni, D, et al. (2001) *Euphytica* 121:145-156, for the proposition that embryonal recombination and germline inheritance of recombined tobacco loci show variable recombination efficiencies, and Vergunst (1998) *Nun. Acids. Res.* 26: 2729-2734, for the unpredictability of using the recombinase system as evidenced by instability of recombinants and phenotypic "escapes." Thus, the Examiner concludes that, without further guidance, it is unpredictable that one skilled in the art would be able to express in a plant a structural gene encoding a compound that is detrimental to the plant, as applicants have provided no guidance on how to eliminate predictably inoperable embodiments.

Applicants respectfully submit that recent work has demonstrated that site-specific recombinases can be nearly 100% efficient. Attached hereto as Exhibit A is Zuo et al. (2001) *Nature Biotechnology* 19: 157-616. In Zuo, all 19 of the *Arabidopsis* lines created using a Cre/Lox mediated excision system underwent excision. Luo et al. (2000) *The Plant Journal* 23(3): 423-430, attached hereto as Exhibit B, shows similar success with FLP/FRT. Instances where the efficiencies of the recombinases were found to be low can be attributed to weak promoters, or, as discussed by Luo, may be due to position effects (Luo at p. 427).

Genes may be silenced due to these position effects, thus negating their expression. These deleterious effects may be overcome by selecting the transgenic lines whose transgenes are not silenced. Such selection is within the ordinary skill in the art. Furthermore, even if a transgenic line were "leaky" and some of the detrimental compound were expressed too early in some plants, such would reduce the yield, and perhaps even kill a percentage of the transgenic plants, but would not render the invention inoperative. Accordingly, applicants respectfully request withdrawal of this rejection.

Claims 9 and 20-24 remain rejected under 35 U.S.C. § 103(a) as being obvious over Kilby (1995), Plant Journal 8: 637-652, in view of Odell, U.S. Patent No. 5,658,772, and Kilby (1993), Trends in Genetics 9: 413-421. The Examiner finds that the term "extracting the compound in economical quantities" is given no patentable weight due to lack of clarity of this claim term. Accordingly, the Examiner reiterates this prior rejection.

As discussed above, applicants respectfully submit that the term "economical quantities" refers to a compound that is produced in sufficient quantities that the value of the extracted compound exceeds the costs associated with standard production and extraction methods.

As applicants have argued previously, applicants do not believe that the term "extracting the compound in economical quantities" is necessary to distinguish from the teachings of Odell, in combination with the Kilby references. While Odell teaches production of barnase under the control of site-specific recombination sequences, Odell does not teach extraction of the barnase itself. Instead, in Odell, barnase is provided in situ to disrupt seed development. It is believed that only small amounts of barnase are needed, and Odell is completely silent about extraction of this detrimental compound. While Odell teaches extraction of DNA and RNA for analysis purposes, this is different from extraction of the resultant barnase protein, which may or may not be present and stable in extractable quantities. Further, because the barnase is used for in situ disruption of the seed development to produce seedless watermelon, extraction of the barnase would be counter to the disclosed use of this detrimental compound. Odell, alone or in combination with the Kilby references, simply fails to teach or suggest production of a detrimental compound and extraction of that same compound. The term "extracting the compound in economical quantities" was added in a prior amendment to differentiate further the present invention from the teachings of Odell, as Odell is completely and utterly silent regarding production of the detrimental compound for extraction as a commercially valuable product. Odell, alone or in combination with the

Kilby references, fails to teach or suggest production and extraction in economical quantities of a detrimental compound.

Because the Kilby and Odell references fail to teach or suggest the use of the claimed constructs for the expression and extraction of economic quantities of the detrimental compound, applicants respectfully request withdrawal of this rejection.

CONCLUSION

The application as amended, is believed to be in condition for allowance.  
Withdrawal of the rejections and passage of the application to issuance is requested.

Respectfully submitted,



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## EXHIBIT A

# Chemical-regulated, site-specific DNA excision in transgenic plants

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We have developed a chemical-inducible, site-specific DNA excision system in transgenic *Arabidopsis* plants mediated by the Cre/loxP DNA recombination system. Expression of the Cre recombinase was tightly controlled by an estrogen receptor-based fusion transactivator XVE. Upon induction by  $\beta$ -estradiol, sequences encoding the selectable marker, Cre, and XVE sandwiched by two loxP sites were excised from the *Arabidopsis* genome, leading to activation of the downstream GFP (green fluorescent protein) reporter gene. Genetic and molecular analyses indicated that the system is tightly controlled, showing high-efficiency inducible DNA excision in all 19 transgenic events tested with either single or multiple T-DNA insertions. The system provides a highly reliable method to generate marker-free transgenic plants after transformation through either organogenesis or somatic embryogenesis.

Keywords: DNA excision, Cre/loxP, marker-free transformants, transgenic plants, XVE

The use of transgenic technology for agricultural purposes has encountered several challenges. One concern is related to the presence in transgenic crop plants of marker genes conferring antibiotic or herbicide resistance. Although no scientific basis has been determined for these concerns, removal of marker genes would likely hasten the public acceptance of transgenic crops.

To this end, several systems for the generation of marker-free transgenic plants have been described. One rather time-consuming approach is to excise or segregate a marker gene from a target gene by sexual crosses or retransformation<sup>1–3</sup>. A second approach is based on overexpression of the isopentenyl transferase (*ipt*) gene<sup>10,11</sup>. In an early attempt, Ebinuma and coworkers developed an *ipt*-based transformation system with low efficiencies (0.5–1.0%)<sup>12</sup>. Recently, these researchers reported an improved marker-excision system using the R recombinase controlled by an inducible promoter, and obtained five marker-free plants from 37 *ipt*-shooty lines<sup>13</sup>. However, the lack of genetic data makes it uncertain whether DNA recombination had occurred in the germplines of these plants. Moreover, this system is not suitable for most economically important crops, which are regenerated through somatic embryogenesis rather than cytokinin-dependent organogenesis. In an independent effort, Kunkel *et al.*<sup>14</sup> placed *ipt* under the control of the GAL4-VP16-glucocorticoid receptor (GVC)-inducible expression system<sup>15</sup> enabling inducible *ipt* expression and regeneration of transgenic plants. Although this method improved the transformation efficiency, the non-plant 35S-GVC and UAS-*ipt* transgenes may also raise public concerns. Additionally, the GVC-*ipt* system can only be used for the limited number of plant species that depend on organogenesis for regeneration.

To develop a reliable system for excision of selectable markers, it is important to distinguish successful DNA recombination events in germline cells from those in somatic cells. Whereas marker excision from somatic cells may be useful for vegetative propagation of transgenic plants<sup>12,13,16</sup>, the technology cannot be applied to most crop plants, which are propagated by seeds. To produce marker-free progeny, successful DNA recombination must occur in gametes or their L2 progenitor cells in the shoot apical meristem<sup>17–20</sup>. Therefore, the

general utilization of a marker excision system depends largely on the DNA excision efficiency in germline cells.

We present here a chemical-inducible, site-specific DNA excision system in transgenic *Arabidopsis* plants, termed CLX (for Cre/loxP DNA excision system), controlled by the XVE system<sup>21</sup>. Compared to previously reported systems, the CLX system is tightly controlled and DNA excision can be induced at high efficiency. More important, this system is useful for all types of explant regeneration either by organogenesis or somatic embryogenesis.

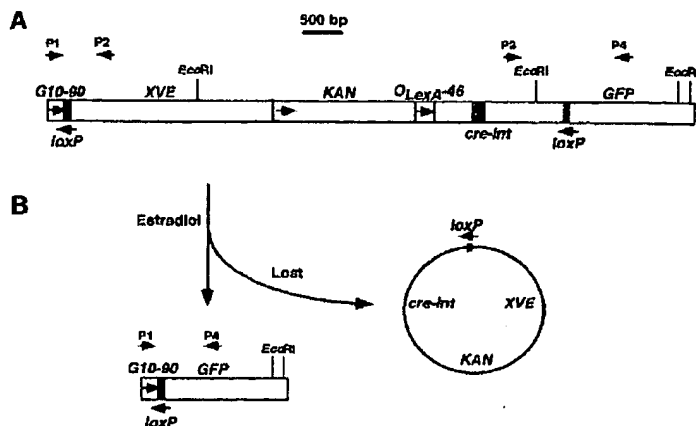
## Results

**A chemical-inducible Cre/loxP DNA recombination system.** Our strategy was to transform and regenerate transgenic plants using any conventional selectable marker, and subsequently to remove the marker from the host plant genome by chemically regulated site-specific DNA excision. The XVE-inducible expression system<sup>21</sup> was chosen to construct the CLX system (Fig. 1). The bacteriophage P1 Cre recombinase, which specifically recognizes loxP sites both *in vivo*<sup>22</sup> and in plant cells<sup>2,23</sup>, was placed under the control of the XVE system. Because the *O*<sub>LexA</sub>-46 promoter has background expression in bacterial cells, the Cre coding sequence was interrupted by a short intron to prevent bacterial expression of cre. The cre-int fusion gene was generated by inserting Intron 5 of the *Arabidopsis* KORI gene<sup>24</sup> between codons 144 and 145 of the cre sequence. Nucleotides 4–6 of the intron (TTG) were mutated to AGT to better match the splicing consensus in *Arabidopsis*. A kanamycin-selectable marker was placed between the XVE and the cre transcription units. These three transcription units were flanked by two loxP sites so that  $\beta$ -estradiol-induced DNA recombination would remove all these components, leading to the activation of the downstream GFP gene by the G10-90 promoter<sup>25</sup>.

The CLX vector pX6-GFP was introduced into *Arabidopsis* by *Agrobacterium*-mediated root culture transformation<sup>26</sup>. Forty-three putative transformed shoots (T<sub>0</sub>) were generated after four to five weeks of culturing on a kanamycin-containing shoot regeneration medium. Five shoots were transferred onto an inductive medium, and the remaining 38 shoots were transferred onto

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**Figure 1.** A schematic diagram of the CLX vector and  $\beta$ -estradiol-induced DNA excision. (A) Structural features of the CLX vector pX6-GFP. (See ref. 21 for details of the XVE system.) Three transcription units are located within the two *loxP* sites: XVE consists of the coding sequence of the XVE hybrid transactivator terminated by the *rbcs* E9 polyA addition sequence, and is activated by the *G10-90* promoter upstream of the *loxP* site; KAN consists of the neomycin transferase II (*NPTII*) gene promoter, the coding sequence of the neomycin transferase II (*NPTII*), and the *NOS* polyadenylation sequence; *cre-int* consists of eight copies of the *LexA* operator sequence fused to the -48 *CaMV35S* promoter, the coding sequence of *Cre* interrupted by an intron and terminated by the *NOS* polyadenylation sequence. Downstream of the second *loxP* site, the *GFP* cDNA was terminated by the *rbcs* 3A polyA addition sequence. Arrows inside squares indicate the direction of transcription. P1 through P4 denote primers used for PCR analysis shown in Figure 2A. Four *EcoRI* sites (used for genomic Southern blot analysis in Fig. 2B) are located at nucleotides 1,798, 5,615, 7,326, and 7,496, respectively. (B) Putative products of the  $\beta$ -estradiol-induced site-specific DNA recombination.

Murashige-Skoog (MS) medium<sup>27</sup>. After induction for two weeks, uniform GFP fluorescence was detected in roots of all five explants cultured on the inductive medium, whereas no GFP expression was observed in explants grown on the noninductive MS medium (15 lines examined). All 43 independent lines were then transferred to soil, and T<sub>1</sub> seeds were obtained from 23 lines including 3 lines (numbers 1–3) previously treated with  $\beta$ -estradiol at the T<sub>0</sub> generation. The death before flowering of the remaining 20 lines was presumably due to poor root growth and development.

**Genetic analysis of putative T<sub>0</sub> recombinants.** T<sub>1</sub> seeds from transgenic lines 1, 2, and 3, treated with  $\beta$ -estradiol in the T<sub>0</sub> generation, were germinated on MS medium and analyzed for GFP expression. The proportion of GFP-positive (GFP<sup>+</sup>) progeny was less than

that expected for a dominant gene (column 2, Table 1). To estimate the recombination frequency, the transgenic locus number was first determined by transferring all GFP-negative (GFP<sup>-</sup>) progeny either to the inductive or the selective medium, and additional T<sub>1</sub> progeny showing either  $\beta$ -estradiol-induced GFP expression or kanamycin resistance (KAN<sup>R</sup>) were identified. It appeared that lines 1 and 3 contained a single transgenic locus, and line 2 contained possibly two transgenic loci (column 3, Table 1). From the number of originally GFP<sup>+</sup> progeny and the transgenic locus numbers, we estimated that DNA recombination had occurred in ~29–66% of germ cells.

In line 2, whereas both transgenic loci appeared to undergo successful DNA recombination upon induction, excision at neither locus was complete. Formation of genetic chimeras in these T<sub>0</sub> transgenic plants could be due to inaccessibility of the L2 progenitor cells to the inducer and inducer instability as well as positional effects of the T-DNA insertions (see below). In lines 1 and 3, T<sub>2</sub> progeny of the putative T<sub>1</sub> recombinants, expressing GFP without the inducer, showed a complete loss of the KAN<sup>R</sup> marker gene and a mendelian segregation for GFP expression in all 17 families tested (Table 1), suggesting complete and precise DNA recombination.

DNA recombination is highly inducible in all tested transgenic lines. To investigate whether the  $\beta$ -estradiol-induced DNA excision occurred in each of the transgenic lines, T<sub>1</sub> seeds from the 20 noninduced transgenic lines (numbers 4–23) were germinated on either the selective or the inductive medium. Four independent lines (numbers 9, 13, 18, and 19) appeared to be nontransgenic escapes based on genetic and molecular analyses. No GFP expression was detected in the remaining 16 transgenic lines when grown on the selective medium, but all lines showed individuals with uniform GFP expression after  $\beta$ -estradiol treatment, indicating that the CLX system in all of these lines was tightly controlled and highly responsive to the inducer. Within each T<sub>1</sub> line, GFP plants were kanamycin-sensitive (KAN<sup>S</sup>) after transfer to the selective medium, indicating that they were wild-type progeny. Based on the segregation patterns of both the selection marker and GFP expression, four lines (numbers 6, 8, 11, and 22) appeared to contain a single transgenic locus whereas six lines (numbers 4, 5, 7, 12, 14, and 16) apparently contain multiple transgenic loci. In these 10 transgenic lines, both the selection marker and the  $\beta$ -estradiol-induced GFP expression segregated in a mendelian manner. The transgenic copy number was inherently difficult to determine in lines

**Table 1.** Genetic analysis of T<sub>1</sub> and T<sub>2</sub> progeny derived from putative T<sub>0</sub> recombinants

Line	T <sub>1</sub> <sup>a</sup>		T <sub>2</sub> (family) <sup>d</sup>		
	GFP <sup>+</sup> /GFP <sup>-</sup> (%) <sup>b</sup>	Segregation of transgene <sup>c</sup>	KAN <sup>R</sup> /KAN <sup>S</sup>	GFP <sup>+</sup> /GFP <sup>-</sup> Heterozygous	GFP <sup>+</sup> /GFP <sup>-</sup> Homozygous
1	18/64 (29.3)	63/19	0/1,274 (8)	941/307 (7)	90/0 (1)
2	33/20 (66.4)	53/0	Variable <sup>e</sup>	Variable <sup>e</sup>	Variable <sup>e</sup>
3	18/26 (64.5)	32/12	0/1,235 (9)	652/211 (6)	240/0 (3)

<sup>a</sup>Grown on MS medium without kanamycin or  $\beta$ -estradiol. GFP expression was examined three to seven days after germination.

<sup>b</sup>Excision efficiency (%): the ratio of observed to projected (based on a 3:1 or 15:1 segregation) GFP<sup>+</sup> progeny.

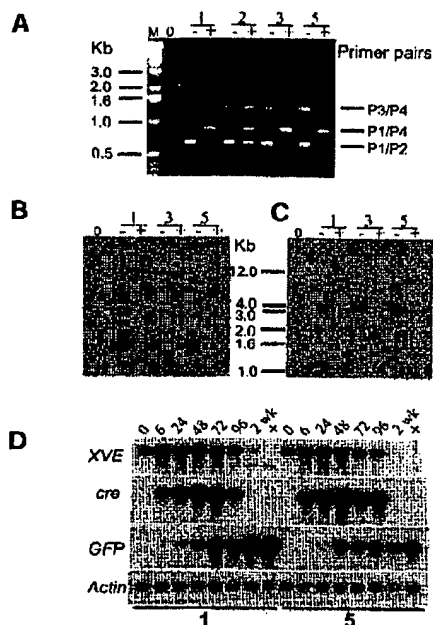
<sup>c</sup>GFP<sup>+</sup> plants were transferred onto a kanamycin-selective or an inductive medium. Additional T<sub>1</sub> progeny that showed KAN<sup>R</sup> or GFP expression were identified. Therefore, the segregation indicates the ratio of the sum of seedlings showing KAN<sup>R</sup> and GFP<sup>+</sup> to those without either trait.

<sup>d</sup>Randomly selected T<sub>1</sub> GFP<sup>+</sup> seedlings were transferred to soil. Seeds were collected from individual T<sub>1</sub> plants and placed on different media. The numbers of tested T<sub>2</sub> families are given in parentheses. KAN<sup>R</sup> / KAN<sup>S</sup> plants were germinated on the kanamycin-selective medium. KAN<sup>R</sup> or KAN<sup>S</sup> phenotypes were scored 7–10 days after germination. GFP<sup>+</sup>/GFP<sup>-</sup> plants were germinated on MS medium only. Heterozygous or homozygous indicates families showing segregation or no segregation, respectively, for GFP expression; therefore, the respective T<sub>1</sub> progenitors were most likely heterozygous or homozygous, respectively, for the transgenic locus. When germinated on the inductive medium, a similar segregation pattern was observed for these families.

<sup>e</sup>Both the kanamycin-selective marker and GFP expression showed a variety of segregation patterns including 15:1 (two families), 3:1 (one family), and no segregation (five families).



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**Figure 2.** Molecular characterization of  $\beta$ -estradiol-induced site-specific DNA excision in transgenic *Arabidopsis* plants. (A) PCR analysis of genomic DNA prepared from wild-type (lane 0) and transgenic plants (all  $T_1$  plants; lines 1, 2, 3, and 5 as indicated on the top) using primers P1 through P4. The expected PCR products from different combinations of primer pairs are indicated on the left. Uninduced transgenic plants (– lanes) were grown on the selective medium. Putative recombinants (+ lanes) were  $T_2$  progeny (grown on MS medium) derived from  $\beta$ -estradiol-treated  $T_0$  (lines 1, 2, and 3) or  $T_1$  (line 5) transgenic plants. M, DNA molecular weight markers. (B, C) Genomic Southern blot analysis. Blots containing digested genomic DNA (3  $\mu$ g) from wild-type and transgenic plants were hybridized with either a GFP probe (B) or a NPTII (C) probe. (D) Northern blot analysis of transgene expression. Two-week-old  $T_1$  plants (lines 1 and 5 as indicated at the bottom) germinated on the selective medium were transferred to the inductive medium and incubated for various time periods (numbered lanes indicate number of hours; 2 wk denotes transgenic plants that were grown on the inductive medium for two weeks; + lanes, same as in part A). Variations in transgene expression in line 5 were presumably caused by the segregation of two transgenic loci among  $T_1$  progeny. Note that the minor band shown in the XVE blot is due to residual signal from the GFP probe.

10, 15, 17, 20, 21, and 23 because of the small population sizes.

To further characterize the system, we analyzed  $T_2$  progeny from eight transgenic lines (numbers 4, 5, 6, 7, 11, 12, 14, and 16) in detail. The remaining eight lines (numbers 8, 10, 15, 17, 20, 21, 22, and 23), which showed  $\beta$ -estradiol-inducible GFP expression in the  $T_1$  generation, were not investigated further.  $T_2$  progeny of the putative  $T_1$  recombinants (treated with  $\beta$ -estradiol and showing GFP expression) showed an excision efficiency similar to that of lines 1, 2, and 3. On the other hand,  $T_1$  nonrecombinants showed a characteristic mendelian segregation pattern for both KAN<sup>R</sup> and  $\beta$ -estradiol-dependent GFP expression (Tables 2 and 3). The above results indicated that the  $\beta$ -estradiol-induced DNA excision occurred in all transgenic lines examined.

Independent DNA recombination in multiple transgenic loci. Segregation analysis suggested that seven transgenic lines (numbers 2, 4, 5, 7, 12, 14, and 16) contained more than one transgenic locus. To address whether all of the transgene copies were excisable upon induction, we selected  $T_2$  families with an approximately 15:1 segregation ratio for KAN<sup>R</sup>:KAN<sup>S</sup>, which should be heterozygous for both

transgenic loci, and tested for the  $\beta$ -estradiol-induced GFP expression. If both copies of the transgene could undergo recombination, the GFP expression phenotype should also segregate in a 15:1 ratio; or a 3:1 ratio if only one copy underwent recombination. In all seven tested transgenic lines,  $\beta$ -estradiol-induced GFP expression showed a segregation pattern similar to that of the KAN<sup>R</sup> selective marker (Table 3). Note that the GFP reporter gene appeared to uniformly express in the inducer-treated transgenic plants as examined under a fluorescence microscope. Some of these plants, however, were genetic chimeras as shown by subsequent genetic (Table 2 and 3) and molecular analyses (see below). Nevertheless, the above results demonstrated that all T-DNA insertions were fully functional for  $\beta$ -estradiol-induced, independent DNA recombination.

**Molecular analyses of  $\beta$ -estradiol-induced DNA recombination.** To characterize the  $\beta$ -estradiol-induced DNA recombination at the molecular level, we performed PCR analysis using primers specific for the excised sequences and flanking nonexcised sequences (see Fig. 1). In a nonrecombinant T-DNA, P1/P2 and P3/P4 will amplify two DNA fragments of 653 and 1,376 bp, respectively; P1/P4 will be unable to amplify a 6 kb DNA fragment under the assay conditions. Upon correct DNA recombination, however, P1/P4 will amplify the rejoined *G10-90-loxP-GFP* sequence (990 bp). In uninduced plants, only the P1/P2 and P3/P4 amplified fragments were detected, indicating that the system is tightly controlled. After induction, three DNA fragments were detected in genomic DNA prepared from transgenic line 2 plants containing two transgenic loci. These three amplification products corresponded to the P1/P2 and P3/P4 fragments in nonrecombinant T-DNA and to the P1/P4 fragment in recombinant T-DNA. However, in the remaining three lines tested (single transgenic locus, lines 1 and 3; multiple transgenic loci line 5) only the P1/P4 fragment was detected (Fig. 2A), indicating complete DNA excision. Among other tested lines, three (single transgenic locus lines 6 and 11 and multiple transgenic loci line 14) showed complete DNA excision, and four (transgenic lines 4, 7, 12, and 16; all with multiple transgenic loci) with incomplete DNA excision.

We performed genomic Southern blot analysis to test whether the excised DNA fragment was reinserted elsewhere in the host plant genome. A GFP probe detected an *EcoRI* fragment with the expected size in uninduced transgenic plants, whereas larger DNA fragments were detected in recombinant plants (Fig. 2B), presumably representing fusion events between *Arabidopsis* genomic DNA and the remaining *G1090-GFP* transgene (see Fig. 1). Using XVE, *cre* (data not shown), and *NPTII* (Fig. 2C) coding sequences as probes, no hybridization signal was detected in recombinant plants, whereas a DNA fragment of the expected size was present in uninduced transgenic plants. This indicates that the excised DNA did not reinsert into the host plant genome. Note that two DNA fragments were detected by the GFP probe in line 5, containing two copies of the T-DNA insert, further demonstrating that both copies were indeed functional (Fig. 2B).

**Table 2. Genetic analysis of  $T_2$  progeny derived from  $T_1$  nonrecombinants with a single transgenic locus<sup>a</sup>**

Line	$T_1$ Heterozygous (family)		$T_1$ Homozygous (family) <sup>b</sup>	
	KAN <sup>R</sup> /KAN <sup>S</sup>	GFP <sup>+</sup> /GFP <sup>-</sup>	KAN <sup>R</sup> /KAN <sup>S</sup>	GFP <sup>+</sup> /GFP <sup>-</sup>
1	203/69 (2)	283/88 (2)	225/0 (2)	161/0 (2)
3	179/67 (2)	278/83 (2)	259/0 (2)	137/0 (2)
6	168/53 (2)	171/56 (2)	179/0 (2)	120/0 (2)
11	250/85 (2)	153/48 (2)	212/0 (2)	120/0 (2)

<sup>a</sup> $T_1$  plants were grown on the selective medium, and  $T_2$  seeds were used in these experiments. See Table 1 footnotes for other technical details.

<sup>b</sup>No GFP expression was detected in any tested KAN<sup>R</sup> plants (200 seedlings from eight families).

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**Table 3. Genetic analysis of T<sub>2</sub> progeny derived from T<sub>1</sub> nonrecombinants with T-DNA insertions in multiple loci<sup>a</sup>**

Line	KAN <sup>R</sup> /KAN <sup>S</sup> (family)	GFP <sup>+</sup> /GFP <sup>-</sup> (family)
2	248/17 (2)	192/12 (2)
4	90/6 (1)	85/7 (1)
5	256/18 (2)	176/11 (2)
7	113/9 (1)	99/7 (1)
12 <sup>b</sup>	115/5 (1)	86/4 (1)
14 <sup>b</sup>	123/5 (2)	177/9 (2)
16	263/18 (3)	366/26 (3)

<sup>a</sup>T<sub>1</sub> plants were grown on the selective medium and the resulting T<sub>2</sub> seeds used in these experiments. See Table 1 footnotes for other technical details.

<sup>b</sup>In these two lines, both KAN<sup>R</sup> and GFP expression appeared to segregate in a ratio greater than 15:1, presumably as a result of linkage between these two transgenic loci.

To monitor the progression of induced DNA recombination, we examined transgene expression by northern blot analysis at different times after  $\beta$ -estradiol treatment (Fig. 2D). The XVE fusion gene was expressed in uninduced plants but not in recombinant plants (+ lanes). The XVE fusion gene, along with the contiguous excisable DNA segment, was lost upon inducer treatment. As expected, XVE expression gradually declined upon extended induction, *cre* expression was tightly controlled by the XVE system without detectable expression in uninduced plants, but strong induction after  $\beta$ -estradiol treatment. Similar to that of XVE, *cre* expression gradually decreased with time, presumably as a result of transgene excision and/or inducer instability (ref. 21). Presumably, this instability, as well as the partial accessibility of L2 progenitor cells to  $\beta$ -estradiol, lead to the formation of genetic chimeras in some transgenic lines. In contrast to the declining XVE and *cre* expression, GFP expression, which is strictly dependent on correct DNA recombination, was detectable after 6 h of induction and progressively increased upon prolonged  $\beta$ -estradiol treatment. Consistent with the above, GFP fluorescence was usually detectable after 12–16 h of  $\beta$ -estradiol treatment.

**Discussion**

Here we describe a tightly regulated and highly efficient site-specific DNA excision system in transgenic *Arabidopsis* plants. This system has several advantages. First, it is tightly controlled by  $\beta$ -estradiol and, moreover, any leaky expression will result in no regeneration of transgenic plants due to the loss of the selectable marker. Second, site-specific DNA excision can be induced at any given time. Third, upon induced DNA excision, all "used" components of the system including the selectable marker and the XVE system itself, will be removed from the host plant genome. This feature is of utmost importance for the generation of marker-free transgenic plants, and for the generation of transgenic plants carrying multiple transgenes. Fourth, upon induced DNA excision, the target gene is permanently activated, a situation that presents key advantages over transiently induced target gene expression (such as the XVE system itself). If desired, the *G10-90* promoter can also be excised by placing a *loxP* site upstream from the promoter, whereas a promoter of interest can be used to control the target gene. Fifth, because the system can use any conventional selectable marker, it is applicable to any transformation method. Finally, because multiple transgene insertions, either linked or independent, occur frequently during plant transformation, the removal of all copies of the selectable marker from the host plant genome is of concern. The CLX system is capable of efficiently excising DNA sequences from multiple T-DNA insertions, whether unlinked or linked.

Compared to the GST-MAT system, in which 14% of the induced-transgenic lines underwent DNA excision<sup>13</sup>, the CLX system appeared to function in all 19 transgenic lines examined. More importantly, we provide compelling genetic evidence showing a high

efficiency (29–66%),  $\beta$ -estradiol-dependent DNA recombination in germline cells, which was unknown for the GST-MAT system. With appropriate improvements in the induction conditions, a higher DNA excision efficiency may be expected.

Based on the KAN<sup>R</sup> and GFP expression phenotypes, each of the 19 tested lines responded to the inducer, and showed uniform somatic GFP expression. Nevertheless, genetic and molecular analyses revealed that some of the treated lines were genetic chimeras, presumably caused by incomplete DNA excision in the L2 somatic progenitor cells because of inducer inaccessibility or instability<sup>21</sup>. This problem may be obviated by repeated applications of the inducer, particularly to the shoot meristem region. In any case, this disadvantage of the CLX system should not detract from its use for regulated, DNA excision in transgenic crop plants. As demonstrated here with *Arabidopsis* lines 1, 2, and 3, primary transformants can be immediately treated with the inducer to obtain T<sub>1</sub> progeny with all possible segregation patterns, allowing the recovery of transgenic plants with the desired genotype. The appropriate T<sub>1</sub> plants containing only the target gene can be used immediately for outcross with commercial varieties.

**Experimental protocol**

**Plasmid construction.** All molecular manipulations were carried out following standard procedures<sup>28</sup>. Pwo DNA polymerase was used in PCR's according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). To construct pX1, the *Pst*I/*Eco*RI fragment of pMM23 (the *Cre* coding sequence) and a PCR-generated fragment (the *NOS* poly A addition sequence fused to a *loxP* site; pMM23 primed by CreF/3'-*lox2* primers, digested with *Eco*RI) were co-ligated into *Pst*I/*Hinc*II digested pBlueScript vector (Stratagene, La Jolla, CA). A GFP cDNA was inserted downstream of the *loxP* site to yield pX-2.

The *cre-int* fusion gene vector pX-3 was constructed by a series of PCR-ligation-PCR reactions. All PCR fragments were treated with T4 DNA kinase before subsequent ligation reactions, which were then directly used for downstream PCR. Appropriate controls with different combinations of PCR primers were included in steps 2 and 3. (1) ligation 1/2: the CreATG/CreQB primed PCR fragment (codons 1–144 of Cre; pX1 as the template) was ligated with a *Ce*lnF/*Ce*lnB amplified PCR fragment containing intron 5 of the *KOR1* gene<sup>24</sup>. Nucleotides 4–6 of the intron (TTG) were mutated to AGT to better match the splicing consensus in *Arabidopsis*; (2) ligation 3/4: CreATG/*Ce*lnB primed ligation 1/2 (Cre 1–144-KOR1 intron 5 fusion fragment) was ligated to CreVF/CreRIB (Val-145 to the end of Cre sequence; pX1 as the template); (3) PCR5: ligation 3/4 was amplified with CreATG/CreRIB primers. The *Eco*RI-digested PCR5 fragment was inserted into *Stu*I/*Eco*RI digested pX2 to yield pX3 consisting of *cre-int-Tnos-loxP-GFP* sequences.

The *G10-90-loxP-XVE* fusion gene (partial XVE sequence) was made by inserting the LoxT1/ERB1359 primed PCR fragment (treated with T4 DNA kinase; pER8 as the template<sup>21</sup>) into the *Sma*I/*Ecl*1361I digested pLIG1090 vector<sup>25</sup> to generate pX4. To construct pX5, the *Mlu*I/*Xho*I fragment of pER10 (containing 3' portion of the XVE transcription unit, the kanamycin transcription unit, and the *O*<sub>LM4</sub>-46 target promoter) and the *Sall*/*Spe*I fragment of pX3 (containing the *cre-int-Tnos-loxP-GFP* sequences) were co-ligated into the *Mlu*I/*Spe*I digested pX4. The CLX vector pX6-GFP was constructed by replacing the *Sse*833T/*Spe*I fragment of pER8 (ref. 21) with that of pX5 (see Fig. 1). Primers used for PCR:

CreF: 5'-CTGGACACAGTCCCGTGTCCGA-3'  
3'-*lox2*: 5'-GAAGATCTATAACTTCGTATAATGCTATACGAAGT  
TATGATCTAGTAACATAGATGACACC-3'  
CreATG: 5'-CCCGTCGACATGTCCAAATTTACTGACCGTA-3'  
CreQB: 5'-CTGGTCGAAATCAGTCCGTTCCGA-3'  
*Ce*lnF: 5'-GTAAGTCTCTTTTCCTTTACTCTTATCAG-3'  
*Ce*lnB: 5'-CTGCCAAATACAGCAAGGCCGAG-3'  
CreVF: 5'-GTTCTGTTCACTCATGGAAATAGCGATC-3'  
CreRIB: 5'-GCCTTTCCCGCATGAATAATATTGATG-3'  
LoxT1: 5'-TAATAACTTCGTATAGCATACATTATACGAAGTTATGAAT-  
TAAATCCGGGCGGAATGAAA-3'  
ERB1359: 5'-GATGAGGAGAGCTGGGCCAGCCG-3'

The pX6-GFP sequence has been deposited in the GenBank database





## RESEARCH ARTICLES

(accession number: AF330636).

Plant materials, growth conditions, and plant transformation. The *Wassilewskija* ecotype of *A. thaliana* was used in all experiments. The selective and inductive media contained MS medium<sup>27</sup> plus kanamycin (50 mg/L) or 17- $\beta$ -estradiol (2  $\mu$ M), respectively. GFP fluorescence was examined using a Zeiss Axioskop fluorescent microscope.

PCR analysis and genomic Southern blot analysis. Genomic DNA was prepared with the Plant DNAeasy Prep Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Approximately 50 ng of genomic DNA were used for PCR. The reactions were subjected to 94°C for 20 s, 60°C for 30 s, and 72°C for 2 min for 50 cycles. Primers for PCR analyses:

P1: 5'-CCATCTCCACTGACGTAAGGGAT-3'

P2: 5'-CTCGTCAATTCCAAGGGCATCGGT-3'

P3: identical to CreF

P4: 5'-TTGTATAGTTCATCCATGCCATG-3'

Genomic Southern blot analysis was carried out following standard methods<sup>28</sup>. Briefly, genomic DNA (3  $\mu$ g) prepared from wild-type and transgenic plants was digested with *Eco*RI, and separated on a 0.8% agarose gel, and blotted onto a nylon membrane (Stratagene). Blots were hybridized with a GFP probe and the other with a *NPTII* probe labeled with the Megaprimer DNA Labeling System (Amersham, Piscataway, NJ).

All other methods, including plant growth and transformation, and RNA manipulations, have been described<sup>21,24</sup>.

## Acknowledgments

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EXHIBIT B

TECHNICAL ADVANCE

## FLP-mediated recombination for use in hybrid plant production

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### Summary

We have studied the feasibility in *Arabidopsis* of using a site-specific recombination system FLP/FRT, from the 2 µm plasmid of yeast, for making plant hybrids. Initially, *Arabidopsis* plants expressing the FLP site-specific recombinase were crossed with plants transformed with a vector containing kanamycin-resistance gene (*npt*) flanked by FRT sites, which also served to separate the CaMV35S promoter from a promoterless *gusA*. Hybrid progeny were tested for excision of the *npt* gene and the positioning of 35S promoter proximal to *gusA*. GUS activity was observed in the progeny of all crosses, but not in the progeny derived from the self-pollinated homozygous parents. We then induced male sterility in *Arabidopsis* plants using the antisense expression of a pollen- and tapetum-specific gene, *bcp1*, flanked by FRT sites. Upon cross-pollination of flowers on the same male-sterile plants with pollen from FLP-containing plants, viable seeds were produced and the progeny hybrid plants developed normally. Molecular analyses revealed that the antisense expression cassette of *bcp1* had been excised in these plants. These results show for the first time that a site-specific recombinase can be used to restore fertility in male-sterile plants, providing an alternative method for the production of hybrid seeds and plants.

**Keywords:** FLP/FRT, site-specific recombination, male-sterility, hybrid, *Arabidopsis*, antisense.

### Introduction

Hybrids of most crop plants yield 10–30% more than pure inbred lines (Mayo, 1980). The most successful crops utilizing hybrids have been corn and rice. In both crops, cytoplasmic male-sterile (CMS) plants have been successfully used on a large scale in hybrid seed production. However, the use of a unique type of cytoplasm has drawbacks because of the increased vulnerability of the plant to insects and pathogens (Levings, 1990). Therefore alternative methods for producing hybrid plants are highly desirable. In corn, detasseling (removal of the male part of line A) and then pollination by line B for hybrid production is an alternative, but detasseling is costly. Neither detasseling nor CMS systems are available for many economically important crops such as wheat, soybean, canola, or barley, which are still bred and grown as inbred varieties.

An alternative to CMS, and detasseling in the case of corn, is the development of male sterility by the selective ablation of tapetal cells, which are essential for the successful development of pollen (Mariani *et al.*, 1990; Moffatt and Somerville, 1988; Tsuchiya *et al.*, 1995; Xu *et al.*, 1995a). Selective ablation of tapetal cells by cell-specific expression of cytotoxic molecules (Mariani *et al.*, 1990) or an antisense gene (Xu *et al.*, 1995a) blocks pollen development, resulting in male sterility. By blocking the expressed cytotoxic molecules through crossing to a plant that expresses an inhibitor of the cytotoxin, Mariani *et al.* (1990, 1992) were able to restore fertility and thus produce hybrid plants. However, methods to restore the fertility for the antisense-caused male sterility have not been developed. In this instance, as well as in the cytotoxic

strategy, site-specific recombinases may provide a versatile method to restore fertility.

Site-specific recombinases are enzymes that recognize specific DNA sequences, and in the presence of two such recombination sites they catalyze the recombination of DNA strands (Ow and Medberry, 1995). In these site-specific recombination systems, recombinases can catalyze excision or inversion of a DNA fragment according to the orientation of their specific target sites. Recombination between directly oriented sites leads to excision of the DNA between them, whereas recombination between inverted target sites causes inversion of the DNA between them. Some site-specific recombination systems do not require additional factors for their function and are capable of functioning accurately and efficiently in various heterologous organisms. For example, FLP/*FRT* from the 2 µm plasmid of *Saccharomyces cerevisiae* (Broach et al., 1982) and *Cre/lox* from *E. coli* phage P1 (Austin et al., 1981) have been shown to catalyze DNA recombination efficiently in plant cells (Bar et al., 1996; Bayley et al., 1992; Dale and Ow, 1991; Kilby et al., 1995; Lloyd and Davis, 1994; Lyznik et al., 1993; Lyznik et al., 1996; Odell et al., 1990; Russel et al., 1992; Sonti et al., 1995; Srivastava et al., 1999).

Here we have studied the feasibility of using a site-specific recombinase, specifically FLP in *Arabidopsis* plants, for making plant hybrids. Using this system in transgenic male-sterile plants we have successfully eliminated the male sterility-causing elements integrated into the host genome by crossing with pollen from a plant expressing the FLP recombinase, yielding hybrid seeds.

In the hybrid/recombinase system described here, male sterility can be induced by any pollen- or tapetum cell-specific gene whose malfunction causes male sterility. This male sterility-causing gene would ultimately be excised from the host genome. This is unlike the *barnase/barstar* hybrid plant system (Mariani et al., 1992), in which the barnase protein (an RNase) causes male sterility, and fertility is restored by complexing to barstar, an inhibitor of the RNase, blocking the barnase activity. In the present study, male sterility was induced in *Arabidopsis* using the antisense of the *bcp1* gene which had previously been shown by Xu et al. (1995b) to induce male sterility in *Arabidopsis*. We were then able to use FLP to excise the antisense *bcp1* gene to restore fertility. Thus the recombination system offers much more flexibility in designing a sterility-fertility restoration system for hybrid seed production.

## Results

### In planta test of efficacy of FLP-mediated recombination

To test the efficacy of FLP recombination activity when expressed in plants, we first obtained two transgenic lines

of *Arabidopsis thaliana* ecotype Columbia using FLP-containing construct pJFLO and the recombination-reporter construct, pFFG, respectively (Figure 1). In the pFFG construct, the presence of a 1.31 kb *npt* fragment flanked by directly oriented *FRT* sites between the CaMV 35S promoter- $\omega$  enhancer and the *gusA* coding region prevented *gusA* transcription. Excision of the blocking sequence (*npt* gene) by FLP will bring together the 35S promoter and the downstream *gusA* reporter gene, giving rise to *gusA* expression.

After *Agrobacterium tumefaciens*-mediated transformation, transgenic plants were selected for their kanamycin resistance and the presence of the gene was confirmed by Southern analysis (data not shown). Homozygous transgenic lines with one single integrated transgene were obtained by selfing and monitoring for segregation for kanamycin resistance. Two transgenic *Arabidopsis* lines transformed with FLP-containing construct pJFLO (P7) and the recombination-reporter construct, pFFG, respectively (Figure 1a,b) were chosen for subsequent experiments.

After cross-pollination between the FLP-expressing plant (P7) and *FRT*-containing plants, hybrid seedlings were harvested and stained for GUS activity. In total, flowers of 24 *FRT*-containing plants were hand-pollinated with pollen from the FLP-expressing plant (P7). Fifty randomly chosen hybrid seedlings from each crossing event were tested for GUS expression. All the progeny seedlings tested stained blue and exhibited a more-or-less uniform expression of the *gusA* gene (Figure 2). In contrast, the progeny of selfed parental *Arabidopsis* plants did not express detectable GUS activity (data not shown). This observation clearly demonstrated the efficient operation of FLP recombinase in catalyzing excisional DNA recombination, indicating that the FLP/*FRT* recombination system functions in *Arabidopsis* plants.

### Expression of antisense *bcp1* gene in *Arabidopsis* causes male sterility

The *Arabidopsis bcp1* gene is active in both diploid tapetum and haploid microspores. It has been shown to be essential for pollen fertility (Xu et al., 1995a). To induce male sterility in *Arabidopsis*, two constructs, *pbcp1:abcp1* and *p35S:abcp1* (Figure 1c,d), containing antisense *bcp1* gene, were introduced separately into *Arabidopsis thaliana* ecotype Columbia using *A. tumefaciens*-mediated transformation. Transgenic plants were screened from two independent transformation events by spraying with Basta. Of 117 primary transgenic plants (64 from *pbcp1:abcp1* transformation and 53 from *p35S:abcp1* transformation), four from the *pbcp1:abcp1* and two from the *p35S:abcp1* transformants were completely male sterile, without the formation of normally elongated siliques and seeds as observed in the wild-type plant

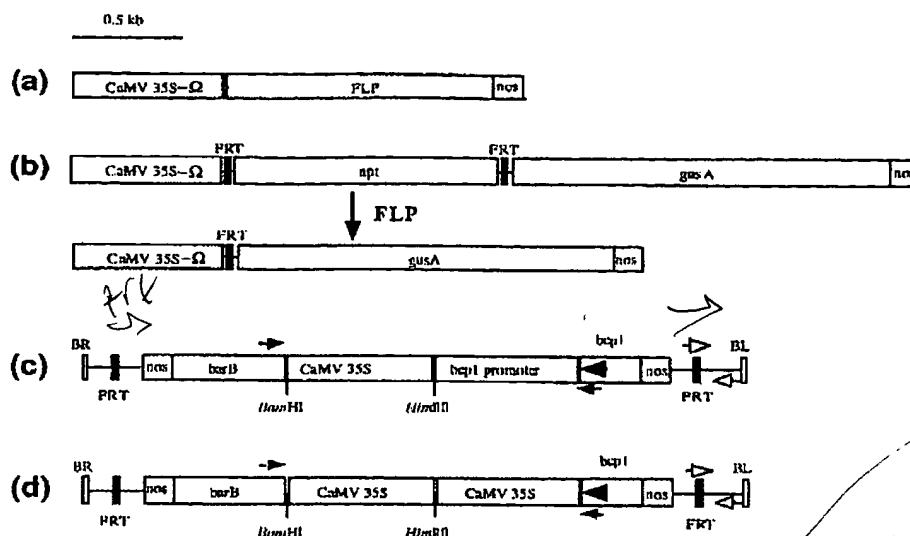


Figure 1. Binary-vector constructs used for *Arabidopsis* transformation.

(a) FLP expression vector pJFLO. The yeast FLP recombinase gene is under the control of CaMV 35S promoter and the omega translational enhancer sequences of TMV (Sleat *et al.*, 1987). Only the FLP cassette within the binary vector is shown here.

(b) FRT recombination-reporter construct pFPG (only the FRT cassette within the binary vector was shown). The *neomycin phosphotransferase* (*npt*) gene flanked by directly oriented FRT sites serves to separate the enhanced CaMV 35S promoter and the *gusA* coding region. Excision of the blocking sequence (*npt* gene) by FLP brings together the 35S promoter and the downstream *gusA* reporter gene, giving rise to *gusA* expression.

(c,d) Constructs with antisense *bcp1*, *pbcp1:abcp1* and *p35S:abcp1*, were used to transform *Arabidopsis* to generate male-sterile plants. Small filled and open arrows in each construct indicate the position of two pairs of primers used for PCR amplification. Large blocked arrows show the orientation of the *bcp1* coding region.

(Figure 3a). All the six male-sterile plants were phenotypically indistinguishable.

PCR assays on genomic DNA extracted from leaves were performed on all six male-sterile plants. Transgenes were identified in all the transformants (Figure 4). Southern analysis using the *bar* gene as probe revealed one transgene insertion in four of the six male-sterile plants, whereas multiple insertions of the transgenes were identified in the other two male-sterile plants (Figure 5a).

#### Removal of antisense *bcp1* gene restores fertility and produces hybrid plants

To determine whether the FLP recombinase can efficiently function to remove the antisense *bcp1* gene between two FRT sites and restore plant fertility, all six hemizygous male-sterile transformants ( $T_0$ ) were cross-pollinated with pollen from FLP-expressing plants (homozygous). If FLP does not function to excise antisense *bcp1* gene, there should still be a 1:1 ratio of fertile to sterile plants, i.e. one-half of the gametes would not contain the antisense gene provided that the transgenic plants have a single-locus integration event. On the contrary, if expression of FLP in the hybrid leads to the removal of the antisense *bcp1* gene, then all the plants in the progeny should be fertile.

Indeed, all progeny from the crosses of FLP-expressing plant to the six hemizygous male-sterile plants produced normal siliques and seeds (Figure 3b). To identify plants with transgene genotypes, we carried out PCR on the genomic DNA from hybrid progeny using primers specific to part of the FRT site and the sequence close to the left border (BL) of T-DNA (Figure 1c,d). First, we ran PCR on the genomic DNA of 120 randomly chosen hybrid progenies from the crosses of an FLP-expressing plant to the four male-sterile lines containing a single transgene insertion. Approximately half (57) gave amplification of the expected 0.3 kb fragment (Figure 6), indicating that 50% of the hybrid plants had the transgene genotypes. More PCR reactions were then carried out on the genomic DNA of hybrid plants from crosses of the FLP-expressing plant to the six male-sterile lines until 60–90 individuals with transgene genotypes were identified in each of the six crosses for further analyses. The fact that all hybrid plants with transgene genotypes grew normally suggests that the excisional recombination catalyzed by FLP in hybrid progenies should have selectively removed the antisense *bcp1* gene and *bar* gene located between two FRT sites, resulting in restoration of fertility.

Genomic analysis of the hybrid progenies with transgene genotypes from all six crosses showed removal of

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the inserted antisense *bcp1* gene (Figures 4 and 5a,b), confirming that the expression of the antisense *bcp1* gene caused the male sterility, and that its removal restored fertility. Thus the crossing event that brought FLP to its target *FRT* sites in male-sterile plants led to the production of fertile hybrid plants. It should be noted that 2% of the hybrid progenies (two out of 90) derived from one male-sterile p35S:*abcp1* transformant ( $M_1$ ) showed a very faint band when their genomic DNA was probed with *bar* gene (Figure 5a, lane  $T_1$ - $M_1$ ). However, the pollen development in these hybrid plants did not appear to be affected by the presence of this small amount of antisense *bcp1* gene product: all the plants are fertile and grow normally.

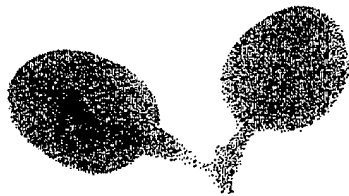
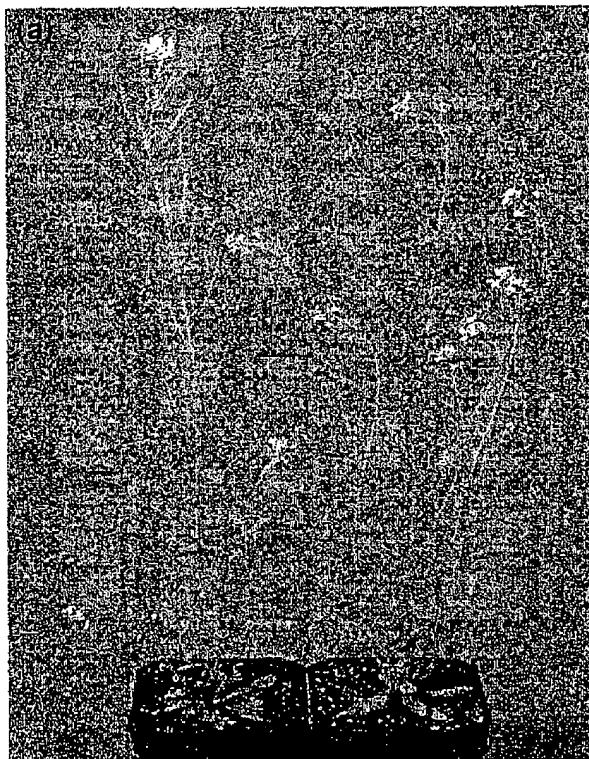


Figure 2. Example of histochemical staining of GUS activity in transformed *Arabidopsis* hybrid seedling obtained from cross-pollination between FLP-expressing and *FRT*-containing plants.



## Discussion

The present study has demonstrated that FLP recombinase of yeast expressed in transgenic *Arabidopsis* can function in excisional DNA recombination. The expression of antisense *bcp1*, driven by its own promoter or by a constitutive 35S promoter, causes male sterility in *Arabidopsis*. Cross-pollination with pollen from a FLP-expressing plant brings FLP recombinase into the male-sterile plant, and the function of FLP results in the removal of the antisense *bcp1* cassette, completely restoring plant fertility in the hybrid progeny. Thus the FLP/*FRT* system is suitable for plant hybrid production.

In recent years a series of genes has been identified that are involved in normal pollen development in many plant species, including maize (Hanson *et al.*, 1989), rice (Xu *et al.*, 1995b; Zou *et al.*, 1994), tomato (Twell *et al.*, 1989), *Brassica campestris* (Theerakulpisut *et al.*, 1991), and *Arabidopsis* (Xu *et al.*, 1995a). The perturbation of their function leads to male-sterile phenotypes (Moffatt and Somerville, 1988; Xu *et al.*, 1995a). Foreign cytotoxic genes that specifically block pollen development can also be used to produce male sterility (Mariani *et al.*, 1990; Tsuchiya *et al.*, 1995). The male sterility caused by either of these systems is expected to be restored to fertility by specific gene deletion with the FLP/

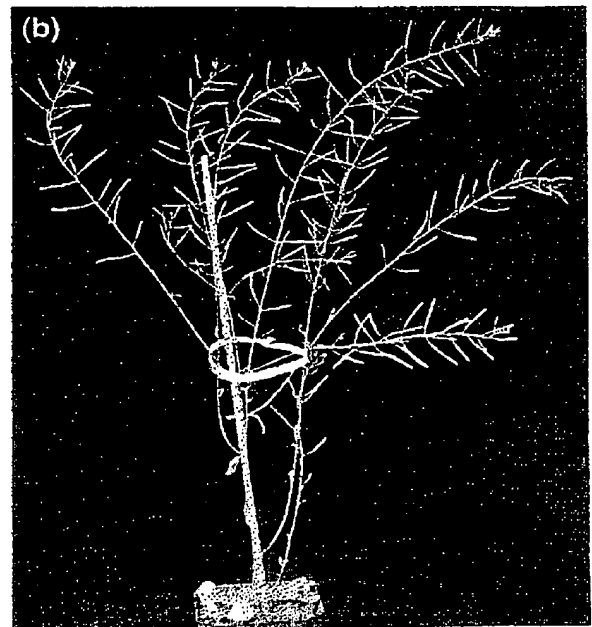
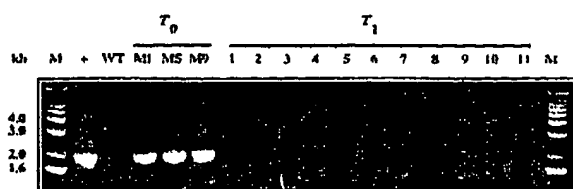
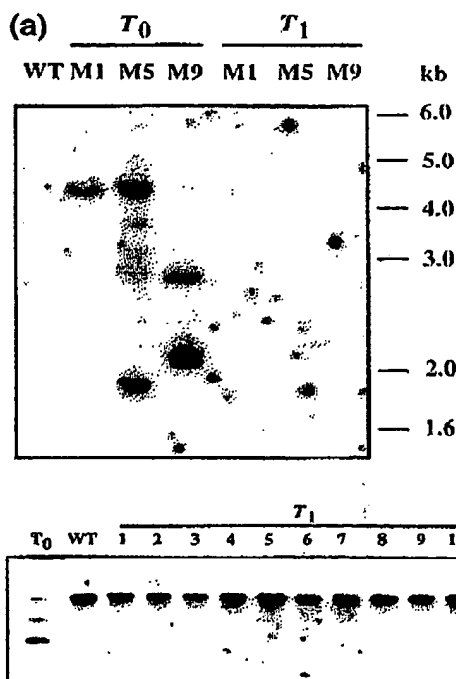


Figure 3. Male-sterile phenotype of transgenic *Arabidopsis thaliana* plants carrying *bcp1* promoter-antisense transgene. (a) Example of siliques from wild-type plant (left) and the transgenic male-sterile plant  $M_9$  (right). Note the shortened siliques in the male-sterile plants. (b) Fertile  $T_1$  hybrid plants obtained after excision of the antisense *bcp1* gene.



**Figure 4.** Ethidium bromide-stained agarose gel showing PCR products amplified from the genomic DNA of three male sterile *T<sub>0</sub>* *Arabidopsis thaliana* plants (*M<sub>1</sub>*, *M<sub>5</sub>* and *M<sub>9</sub>*) and 11 randomly chosen fertile hybrid *T<sub>1</sub>* plants with transgene genotypes. Primers designed for PCR amplification of a DNA fragment between *bcp1* and *bar* genes were as described in Experimental procedures and shown in Figure 1(c,d). Negative control, wild-type plant (WT); positive DNA control, PCR product of the *pbcpl:abcp1* construct (+). A molecular weight ladder (M) is shown.



**Figure 5.** Southern blots of DNA and *bcp1* antisense gene.

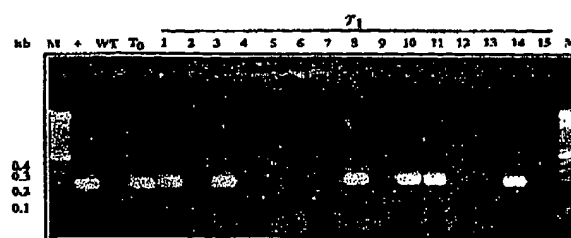
(a) Southern blot of DNA of three male-sterile *T<sub>0</sub>* plants and fertile hybrid *T<sub>1</sub>* progeny plants from each of the *T<sub>0</sub>* male-sterile plants. Genomic DNA was digested with *Bam*HI and the *bar* gene was used as the probe. *T<sub>1</sub>* progeny plants with transgene genotypes correspond to *T<sub>0</sub>* parents as labeled.

(b) Example of Southern blot of *bcp1* antisense gene. The genomic DNA of male-sterile *T<sub>0</sub>* plant M9 and its 11 fertile hybrid *T<sub>1</sub>* progeny plants was digested with *Hind*III; *bcp1* was used as the probe. The antisense in *T<sub>0</sub>* was present as the two lower molecular-weight bands. The endogenous *bcp1* was revealed in *T<sub>0</sub>* as well as all *T<sub>1</sub>* progeny with transgene genotypes.

**FRT recombination system.** This provides a much more flexible method for the production of hybrid plants by genetic engineering strategies.

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**Figure 6.** Ethidium bromide-stained agarose gel showing PCR products amplified from the genomic DNA of one male-sterile *T<sub>0</sub>* *Arabidopsis thaliana* plant (*M<sub>1</sub>*) and its 15 fertile hybrid *T<sub>1</sub>* plants with transgene genotypes.

Primers designed for PCR amplification of a DNA fragment between the second *FRT* site and the left border (BL) of T-DNA were as described in Experimental procedures and shown in Figure 1(c,d). Negative control, wild-type plant (WT); positive DNA control, PCR product of *p35S:abcp1* construct (+). A molecular weight ladder (M) is shown.

Our results also indicate that both a constitutive and a tissue-specific promoter can drive antisense gene expression and consequently result in male sterility. This implies that an inducible promoter system could be used for driving expression of antisense or cytotoxic foreign genes at will. Thus the genes controlling male sterility could be kept silent in transgenic plants during seed multiplication, and then turned on to produce male-sterile plants for use in hybrid seed production. Such regulation of the expression of foreign genes in plants has been achieved with promoters responsive to environmental stimuli (Ainley and Key, 1990; Kyoizuka *et al.*, 1993; Lyznik *et al.*, 1995) or synthetic chemicals (Ayoma and Chua, 1997; Caddick *et al.*, 1998; Gatz, 1997; Mett *et al.*, 1993; Ward *et al.*, 1993).

FLP recombinase has been successfully introduced into tobacco and shown to function efficiently in the progeny of crosses made between primary transformed tobacco plants (Kilby *et al.*, 1995; Lloyd and Davis, 1994). The results presented here demonstrate that in *Arabidopsis*, FLP recombinase can also be constitutively expressed and can catalyze DNA recombination efficiently. Previous efforts to obtain transgenic *Arabidopsis* plants able to constitutively express functional FLP recombinase were unsuccessful. FLP recombinase showed either no activity in plants (Lloyd and Davis, 1994) or poor activity (Sonti *et al.*, 1995), or functioned only when using an inducible heat-shock promoter (Kilby *et al.*, 1995). This difference might relate to the instability of the FLP recombinase expressed in transgenic *Arabidopsis* due to gene silencing or effects of the positions of the transgenes inserted in the host genome (Matzke and Matzke, 1998). The structure of the FLP gene itself may also play an important role. In our studies we used the FLP gene possessing the consensus sequence for plants around the ATG translation initiation codon, i.e. AACAAATG (Joshi, 1987; Lütcke *et al.*, 1987). This is in contrast to the consensus sequence found in animals, i.e. CACCATG, which is also the case for the original FLP

gene (Hartley and Donelson, 1980). The use of the *FLP* gene with the plant consensus sequence around the ATG codon may have an exceptionally positive impact on the correct expression of FLP in transgenic *Arabidopsis*, resulting in its high efficiency in catalyzing DNA recombination.

In the present study we also observed that about 2% (two out of 90 analyzed) of the hybrid progenies derived from one male-sterile p35S:*abcp1* transformant ( $M_1$ ) showed a very faint band when their genomic DNA was probed with the *bar* gene (Figure 5a, lane  $T_1-M_1$ ), although pollen development in these hybrid plants did not appear to be affected by the presence of this small amount of antisense *bcp1* gene product, and the plants are fertile and grow normally. This suggests that in some of the hybrid plants derived from this male-sterile line, the FLP excision of the foreign gene between two *FRT* sites is not complete in all cells. One possible explanation could be that DNA methylation in the *FRT* sites hinders the binding of the FLP protein, resulting in the failure of DNA excision. Environmental factors could also be responsible for the failure of DNA excision. Another possibility could be that, in some cells, the excised antisense *bcp1* gene cassette occasionally integrated again into the host genome, but at a different location than its original site. In any case, further work is needed to determine why a small portion of the hybrid plants derived from one particular male-sterile line do not have complete FLP-mediated DNA excision, whereas excision was complete in all the hybrid plants derived from the other five male-sterile lines.

In conclusion, we have shown the constitutive expression of site-specific recombinase FLP in *Arabidopsis*. Moreover, the expressed FLP in transgenic *Arabidopsis* was completely functional and capable of excising a male sterility-causing antisense gene when crossed with a male-sterile plant, producing fertile hybrid seeds. While this system was tested in *Arabidopsis*, it remains to be seen how site-specific recombinase will perform in agriculturally important crop plants. We have already introduced the site-specific recombination systems into rice, and the preliminary results are very promising. The application of the FLP/*FRT* system in producing hybrid rice is currently under way. We believe that the site-specific recombination systems can be successfully applied in crop plants as a useful alternative method for hybrid seed production.

### Experimental procedures

#### Isolation of *bcp1* gene and its 5' region

The *Arabidopsis bcp1* gene was cloned by PCR amplification from the ecotype Columbia genomic DNA. Primer (5'-GTCGTCGTG-GTTGCCCTCG-3') specific to the 5' region and primer (5'-CGA-CGACCGCAGAGACGCC-3') specific to the 3' region of the *bcp1* coding sequence were used for the amplification of a 0.3 kb fragment, which was then cloned into the PCR-Script SK(+) vector.

Nucleotide sequence of the PCR-amplified fragment corresponded to the sequence data published by Xu *et al.* (1995a) for the *bcp1* gene isolated from *A. thaliana* ecotype Landsberg erecta. A 0.8 kb region of the 5' regulatory region of the *bcp1* gene was isolated by screening an *A. thaliana* ecotype Columbia genomic library with the PCR-amplified *bcp1* gene as probe.

#### Plasmid constructions

Plasmids pJFLO (*FLP* gene expression construct) and pFFG (*FRT* recombination-reporter construct), illustrated in Figure 1(a,b) were essentially as described by Bar *et al.* (1996) with the following modifications: the FLP coding region was amplified by PCR from pOG44 (Stratagene, La Jolla, CA, USA) using the primers 5'-CTGCAGCCCACTCGACAAACATGCCACAATTG-3' and 5'-TTATGCTTAAATCCCGGTTATATGCGTCT-3', specific to the 5' and 3' ends of the FLP-coding region, respectively. These primers were designed to direct a plant optimal translational modification sequence AACA immediately adjacent upstream of the ATG initiation codon, as well as creating *SacI* and *SmaI* sites at the 5' and 3' ends of the amplified FLP product (termed FLPO), enabling its insertional replacement of FLP in pJFL (Bar *et al.*, 1996), generating pJFLO. Two antisense constructs, p35S:*abcp1* and p*bcp1:abcp1* (Figure 1c,d), were prepared as follows: the PCR-amplified *bcp1* gene was released as a *PstI*-*SacI* fragment and fused in the reversed orientation with the CaMV35S promoter to replace the *gusA* coding sequence in pFFG (Bar *et al.*, 1996). The resulting 35S promoter-antisense *bcp1* fusion fragment was released and ligated into the *HindIII* and *EcoRI* sites of the binary vector pSB11 (Komari *et al.*, 1996). One *FRT* sequence was then inserted into the *EcoRI* site, while another *FRT* site together with a selectable marker *bar* gene (under the control of 35S promoter) was ligated into the *HindIII* site of this plasmid, giving rise to the p35S:*abcp1*. To obtain p*bcp1:abcp1*, the pSB11 binary vector, containing the 35S promoter-antisense *bcp1* fusion fragment and one *FRT* site, was cut with *HindIII* and *SacI* to release the 35S promoter. The *bcp1* promoter was then inserted into the *HindIII* and *SacI* sites followed by the ligation of the second *FRT* site as well as 35S promoter-driven *bar* gene into the *HindIII* site. The correct orientation of the gene in the plasmid constructs was confirmed either by sequencing or by restriction analyses. All the expression constructs were mobilized into *Agrobacterium tumefaciens* strain LBA404 by conjugation with a helper plasmid pRK2013 (Koncz and Schell, 1986).

#### Plant maintenance and transformation

*Arabidopsis thaliana* ecotype Columbia plants were used for transformation experiments. Seeds were sown into plastic pots containing MetroMix 360 soil mix. After 2 days incubation at 4°C, plants were grown in growth chambers at 24°C, 16 h light/8 h dark photoperiod, and with 60–80% relative humidity. Plants were transformed by vacuum infiltration essentially according to Bechtold *et al.* (1993). Transgenic plants were selected on medium containing kanamycin sulfate (50 µg mL<sup>-1</sup>) or by spraying a 0.5% of Basta solution on the  $T_0$  transformants. Green plants surviving the antibiotic or herbicide treatment were retained for further analyses.

#### Cross-pollination

Flowers that contained pistils covered by sepals and petals were hand-pollinated when the stamens were about half as high as the

pistils and anthers were green and not shedding pollen. The FLP-transformed plants were used as a source of pollen that was collected with cotton buds from open flowers. The cross-pollinated flowers were marked and all lower siliques on the same plant were removed. After an additional 3 weeks the seeds were collected, dried, and then planted for the production of hybrid progeny seedlings.

#### Staining for GUS activity

GUS activity in hybrid progeny seedlings obtained from cross-pollination was assayed by histochemical staining with 1 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-gluc, Biosynth AG, Staad, Switzerland) as described by Jefferson (1987). Whole seedlings were incubated at 37°C overnight in 100  $\mu$ l reaction buffer containing X-gluc. Prior to photography seedlings were destained in 70% ethanol.

#### DNA extraction and analysis

Genomic DNA was extracted from about 200–500 mg fresh leaves, essentially as previously described (Luo *et al.*, 1995). DNA (5  $\mu$ g) was digested with *Hind*III or *Bam*HI according to the supplier's instructions (Biolabs). Fragments were size-separated through a 0.8% (w/v) agarose gel and blotted onto a Hybond-N<sup>+</sup> membrane (Amersham). The DNA fragment used as a probe was radiolabeled by random priming with a kit from Amersham, and the Southern blots were processed as described by Sambrook *et al.* (1989).

#### Polymerase chain reaction

The two primers designed to amplify a DNA fragment between *bcp1* and *bar* genes in the antisense *bcp1* construct were as follows: 5'-GTCGTCGTGGTTGCCCTCG-3' corresponding to the 5' end of the *bcp1* gene and 5'-CGGCGGATGTCGGCCGGG-3' corresponding to the 5' end of the *bar* coding region (Figure 1c,d). Another pair of primers designed to amplify a DNA fragment between the second *FRT* site and the left border (BL) of T-DNA were as follows: 5'-GGAAGTTCGGAATGATCTCC-3' corresponding to part of the *FRT* site and its adjacent region, and 5'-GATGAAGTGACAGATAGCTGG-3' corresponding to the sequence close to the left border (BL) of T-DNA (Figure 1c,d). The reaction mixtures (25  $\mu$ l total volume), overlaid with a drop of mineral oil, consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl<sub>2</sub>, 0.1% (w/v) Triton X-100, 200  $\mu$ M each of dATP, dCTP, dGTP, and dTTP, 0.5  $\mu$ M of each primer, 0.2  $\mu$ g of template DNA, and 1 unit of Taq DNA polymerase (Qiagen). Amplification was performed in a Perkin-Elmer Cetus thermal cycler programmed for 25 cycles of 1 min at 94°C (denaturation); 2 min at 55°C (hybridization); 3 min at 72°C (elongation); and a final elongation step at 72°C for 10 min. PCR products were separated in a 0.8% or 1.5% (w/v) agarose gel and detected by staining with ethidium bromide.

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